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Identification of proliferin mRNA and protein in mouse placenta

(placental hormone/placental lactogen/prolactin-related protein)

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ABSTRACT **Proliferin is a recently described, prolactin-related protein whose mRNA appears in several murine cell lines during active growth. We have surveyed a number of mouse organs or tissues for the presence of mRNAs that hybridize to cloned proliferin cDNA. Of the tissues tested, only the placenta yielded proliferin-related mRNA. This placental RNA is about 1 kilobase in length, increases sharply between days 8 and 10 of pregnancy, and then gradually declines through day 18. It is more abundant in RNA extracted from the fetal, compared to the maternal, part of the placenta. From a cDNA plasmid library prepared from poly(A)$^+$ placental RNA, two types of proliferin-related clones were isolated, differing in intensity of hybridization to proliferin cDNA. By nucleotide sequence analysis, a strongly hybridizing clone was found to be nearly identical to the proliferin cDNA clone isolated from a library prepared from mRNA of a growing mouse fibroblastic cell line. Using an antiserum prepared against a synthetic proliferin fusion protein, we show that proliferin is secreted as a glycoprotein by minced placental tissue and that it differs from mouse placental lactogen. We conclude that proliferin is a placental hormone that is synthesized in certain mouse cell lines during active growth. Its function during pregnancy and during the growth of cultured cells is presently unknown.**

In the course of a study of gene activation during the growth of murine BALB/c 3T3 cells in culture, Linzer and Nathans (1) identified a cloned cDNA (designated PLF-1) that hybridized to a 1-kilobase mRNA present only in growing cells. This RNA appeared in the pre-S phase of growth stimulation and reached its peak level at the start of DNA synthesis. The nucleotide sequence of the PLF-1 cDNA revealed a single open reading frame encoding a protein of about M_r 24,000 with clear homology to preprolactin (2). Because of its similarity to prolactin and its association with proliferating cells, the encoded mature protein was called proliferin or PLF.

As part of our investigation of the possible role of proliferin in cell proliferation, we wanted to determine the site of synthesis of this protein in the intact animal by screening a series of mouse organs and tissues for the presence of proliferin mRNA. Here we report that of the tissues tested, proliferin mRNA was found only in placenta. Minced placentas secreted immunologically detectable proliferin, which is shown to differ from the previously identified murine placental lactogen (mPL) (3). We conclude that proliferin is a placental hormone that is also associated with the growth of a number of mouse cell lines in culture.

MATERIALS AND METHODS

Preparation and Analysis of RNA. Placentas were isolated from Swiss Webster and BALB/c mice (Charles River Breeding Laboratories), frozen rapidly in dry ice/ethanol or in liquid nitrogen, and stored at −70°C. (Swiss–Webster mice were mated locally and timed from the day of appearance of a vaginal plug; BALB/c mice were mated prior to shipment by the supplier.) In some cases placentas were separated grossly into a maternal component (decidua basalis) and a fetal component (labyrinth, cytotrophoblast, and giant cells). Pituitary glands and fetuses were isolated from Swiss–Webster mice, and adult brain, kidney, liver, ovary, fetal brain, and whole fetuses were isolated from BALB/c mice. For preparation of RNA, tissues were extracted with guanidinium thiocyanate, and poly(A)$^+$ RNA was purified as described (1, 2). To test for the presence of proliferin-related RNA, total RNA from the above tissues was electrophoresed in formaldehyde gels (4, 5), transferred to nitrocellulose filters (6), and hybridized to nick-translated (7) probes as described (1).

cDNA Cloning. Poly(A)$^+$ RNA from late-gestation BALB/c placentas was reverse-transcribed in a reaction mixture containing 5 μg of poly(A)$^+$ RNA, 100 mM Tris·HCl (pH 8.3 at 42°C), 100 mM KCl, 6 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 0.5 mM dCTP, 100 nM (10 μCi; 1 Ci = 37 GBq) [α-^{32}P]dCTP, 100 μg of oligo(dT)$_{12-18}$ (Collaborative Research, Waltham, MA) per ml, 500 units of RNasin (Promega Biotec, Madison, WI) per ml, and 75 units of reverse transcriptase (Life Sciences, St. Petersburg, FL) in a final volume of 50 μl. Second-strand synthesis was carried out as described by Gubler and Hoffman (8). The resulting double-strand cDNA was modified by the addition of homopoly(dG) tails and inserted into pBR322 at the *Eco*RV site, modified with homopoly(dC) tails (8). Hybridized vector-cDNA was used to transform *Escherichia coli* MM294 cells (9) to ampicillin resistance (10), and bacterial transformants were screened for the presence of proliferin-related plasmids as described (2). Hybridizing colonies were then selected for the preparation of plasmid DNA (11).

DNA Sequencing. The insert from the placental cDNA clone PLF-2 was subcloned into the M13 vector mp18 (12) and propagated in *E. coli* JM105 cells. The subclones were sequenced by the dideoxy chain termination method (13), and the reaction products were resolved on acrylamide/urea gels (14). Gels were fixed in 10% acetic acid prior to drying and exposure to Kodak XAR film. The complete sequence on one strand was determined by priming at several locations with M13, 17-base-pair (bp) primer (Collaborative Research) or with synthetic oligonucleotides complementary to sequences within the cDNA insert.

Immunoprecipitation and Protein Gel Electrophoresis of Placental Proliferin. For immunization, proliferin was prepared as a fusion protein with bacterial resolvase encoded by the *gamma-delta* transposon of *E. coli* (15). The construction of the recombinant plasmid containing the entire proliferin

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Abbreviations: bp, base pair(s); mPL, murine placental lactogen.

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cDNA and its expression in bacteria will be described elsewhere. Rabbit antisera were raised by periodic injection of gel-purified proliferin-resolvase fusion protein in Freund's adjuvant. The resulting serum was shown to react with the fusion protein. Antiserum against mPL has been described (16). For analysis of placental proliferin, placentas from BALB/c mice at about day 14 of pregnancy were minced and incubated at 37°C in methionine-free Dulbecco's modified Eagle's medium with 0.02 M Hepes buffer (pH 7.4). After a 10 min equilibration period, tunicamycin to 2 μg/ml was added to one of two samples, and 20 min later 250 μCi of [^{35}S]methionine was added to both samples. Eight hours later the samples were centrifuged to separate cells and culture fluid, and protein of each was analyzed by immunoprecipitation (17) and $NaDodSO_4$/polyacrylamide gel electrophoresis (18).

Amino Acid Sequence Analysis of mPL. Purified mPL (3) was subjected to NH_2-terminal sequencing by step-wise Edman degradation in an Applied Biosystems (Foster City, CA) protein microsequencer. We are grateful to Ned Siegel of the Monsanto Company for this analysis.

RESULTS

Survey of Mouse Tissues for Proliferin mRNA. RNA samples prepared from mouse liver, kidney, ovary, pituitary gland, placenta, fetal brain, and whole fetus were tested for proliferin-related mRNA by electrophoresis and hybridization to plasmid PLF-1 (2). Proliferin-hybridizing sequences were detected only in RNA from placenta, where it was found as a moderately abundant RNA of about 1.0 kilobase (Fig. 1*A*). This RNA is the same size as proliferin mRNA found in BALB/c 3T3, C3H 10T½, and Krebs ascites carcinoma cells (19). The level of the proliferin-related RNA increased abruptly from day 8 to day 10 of pregnancy and then decreased slowly through day 18 (Fig. 1*C*). To determine whether the proliferin-related RNA is from the fetal or maternal part of the placenta, placental tissue from day 17 of pregnancy was separated grossly into fetal and maternal components, and RNA from each of these placental fractions was hybridized to PLF-1. Both preparations contained a 1-kilobase RNA that hybridized to the proliferin probe (Fig. 1*B*), but this RNA was more abundant in the fetal sample than in the maternal sample, suggesting that the fetal component is a major source of proliferin-related RNA.

Isolation and Nucleotide Sequence of a Proliferin-Related cDNA from Placental RNA. In order to clone proliferin-related cDNA derived from placental RNA, poly(A)$^+$ RNA from late gestation placentas of BALB/c mice was transcribed into cDNA and cloned into pBR322. Approximately 1500 bacterial colonies containing recombinant plasmids were screened for proliferin-hybridizing sequences. Replica filters of the colony arrays were prepared and hybridized to PLF-1 DNA. Both strongly and weakly hybridizing colonies were seen, suggesting extensive and slight sequence homology to PLF, respectively. Of the 1500 colonies screened, 7 hybridized strongly and 4 weakly; therefore, both types must be derived from moderately abundant mRNAs. Representatives of each colony type were picked and rescreened, and plasmid DNA was purified from each of them. Initial analysis with a series of restriction endonucleases indicated that the strongly hybridizing clones are similar to each other and are also similar, if not identical, to PLF-1; the weakly hybridizing clones are also similar to each other but have restriction patterns different from those of PLF-1. A detailed analysis of the structure of the weakly hybridizing cDNA clones will be presented elsewhere.

To determine the precise relationship of the strongly hybridizing cDNA clones to PLF-1, we sequenced the cDNA insert from one of the placental clones, designated PLF-2.

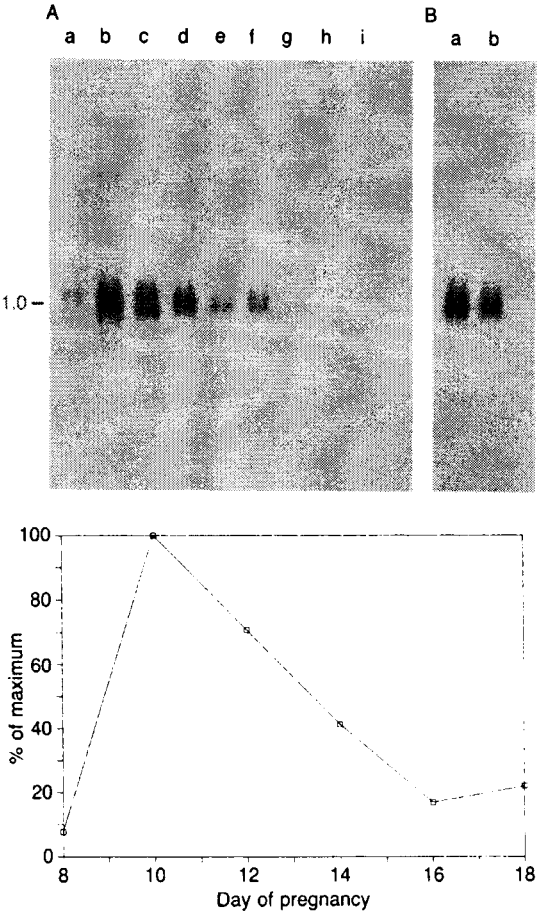

FIG. 1. Expression of proliferin-related RNA in mouse placenta. Total cellular RNA was isolated from mouse tissues, electrophoresed, blotted, probed with nick-translated PLF-1 [^{32}P]cDNA, and autoradiographed. (*A*) RNA prepared from placenta on day 8 of gestation (lane a), day 10 (lane b), day 12 (lane c), day 14 (lane d), day 16 (lane e), and day 18 (lane f), from adult brain (lane g), from fetal brain (lane h), and from whole fetus (lane i). (*B*) RNA prepared from fetal placenta, consisting of labyrinth, cytotrophoblast, and giant cells (lane a) and maternal placenta (decidua basalis; lane b), dissected on day 17 of pregnancy. (*C*) The level of proliferin-related placental RNA during pregnancy. The total optical density of each hybridizing region around 1 kb in *A* was determined with a Loats image-analysis system. The results are given as the percentage of maximum optical density versus day of pregnancy.

The cDNA insert in PLF-2 is 850 bp in length and matches almost exactly the sequence of PLF-1 (Fig. 2). PLF-2 cDNA has an additional 62 bp 5′ to the coding sequence and lacks 3 bp at the 3′ end compared to PLF-1 cDNA. The difference at the 3′ end may be due to the heterogeneity of the poly(A) addition site, as noted earlier (2). The two cDNA clones also differ at five positions in the coding region; each of these differences represents a simple substitution, resulting in predicted amino acid changes in four of the five cases. Since the signal sequence is likely to end at Ser-29 (2), three of the amino acid substitutions (at positions 67, 107, and 117) are in the encoded mature protein. Curiously, all of the amino acid differences involve threonine and serine residues in either PLF-1 or PLF-2, although none of these differences are found at potential glycosylation sites (2). Although the functional significance of this finding is not known, it does encourage speculation about possible differences in phosphorylation sites in PLF-1 vs. PLF-2.

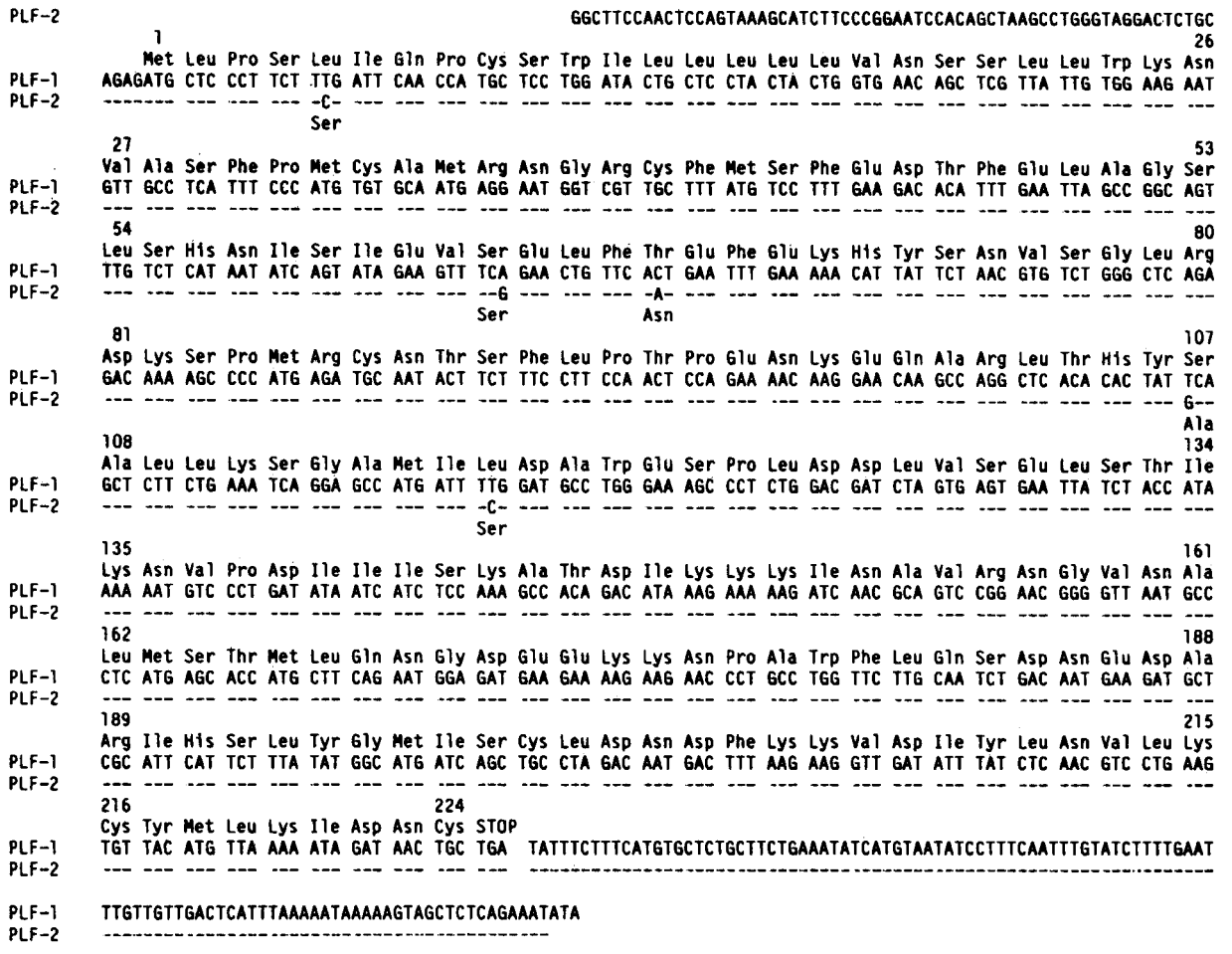

FIG. 2. Sequence comparisons of proliferin clones. The sequence of the placenta-derived cDNA clone PLF-2 is presented along with the BALB/c 3T3 cell-derived clone PLF-1 (2). Numbers refer to the amino acid positions in the unprocessed proteins. A dash indicates nucleotide identity.

Secretion of Proliferin by Placental Tissue and Comparison with mPL. To determine whether proliferin is secreted by the placenta, as predicted from its inferred amino acid sequence, we incubated minced placentas from 14-day pregnant BALB/c mice with [^{35}S]methionine and immunoprecipitated labeled soluble proteins from the medium and from the tissue with antiserum prepared against a recombinant proliferin fusion protein. The antiserum precipitated a protein of about 30 kDa from the postincubation medium and a protein of about 28 kDa from the tissue homogenate (Fig. 3). Pretreatment of the placental tissue with tunicamycin, an inhibitor of N-linked protein glycosylation (20), led to the appearance of an immunoreactive protein with increased electrophoretic mobility consistant with reduction of its M_r to 22,000, the predicted size of mature unmodified proliferin. We conclude that proliferin is secreted by the placenta as a glycoprotein.

To determine whether proliferin is related to the 23-kDa mPL described by Colosi *et al.* (3), we compared the placental protein precipitated by anti-mPL serum with that precipitated by anti-proliferin antiserum. As seen in Fig. 3, anti-mPL antiserum precipitated from the medium a protein of about 23 kDa after incorporation of [^{35}S]methionine in the absence or presence of tunicamycin, thus distinguishing mPL and proliferin. Similarly, by radioimmunoassay with anti-mPL antiserum (16) we were unable to detect the proliferin that was secreted by monkey cells infected with a simian virus 40-proliferin recombinant virus. Finally, the NH_2-terminal amino acid sequence of purified mPL was determined by sequential Edman degradation (21). This sequence —Leu-Pro-Asn-Tyr-Arg-Leu-Pro-Thr-Glu-Ser-Leu-Tyr-Gln-Arg-Val-Ile-Val-Val-Ser—shows no homology with the predicted amino acid sequence of proliferin.

DISCUSSION

The general conclusion of the studies reported in this communication is that proliferin is a murine placental hormone or growth factor made in the fetal component of the placenta and secreted as a glycoprotein. Proliferin mRNA appears to be a major species of placental mRNA in mid- and late-pregnancy; therefore, the protein is likely to be a major secretory product during this period. Proliferin is not the mPL characterized by Talamantes and his colleagues (3). However, it is possible that the 50-kDa midpregnancy lactogen described by these authors (22) is a highly glycosylated form of proliferin, not recognized by our antiserum. Since proliferin is structurally similar to mammalian prolactins (2), we anticipate that it will have prolactin-like activities, including cell proliferative effects. Therefore, proliferin may be a growth factor acting on maternal and/or fetal tissues. The high level of expression of the proliferin gene in growing cells in culture (1) suggests that it also may serve as an autocrine growth factor in nonplacental cells in culture, perhaps related to the immortality of these cell lines. The availability of cDNA clones and

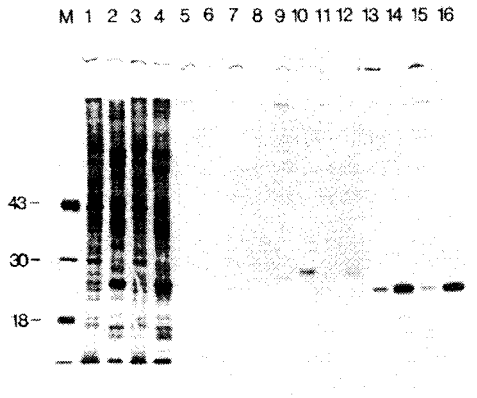

FIG. 3. Autoradiogram after electrophoresis of placental proteins labeled with [^{35}S]methionine. Lanes: M, molecular weight standards of 43, 30, and 18 kDa; 1–4, total labeled proteins; 5–8, proteins reacting with normal rabbit serum; 9–12, proteins reacting with anti-PLF rabbit antiserum; 13–16, proteins reacting with anti-mPL rabbit antiserum; 1, 5, 9, and 13, tissue protein; 2, 6, 10, and 14, proteins appearing in the medium; 3, 7, 11, and 15, tissue proteins synthesized in the presence of tunicamycin at 2 μg/ml; 4, 8, 12, and 16, medium proteins synthesized in the presence of tunicamycin at 2 μg/ml; 1–4 and 13–16, photograph of a film exposed for 5 days; 5–12, photograph of a film exposed for 20 days.

purified proliferin via recombinant viruses or plasmids should allow a test of these hypotheses.

The sequence differences found between the PLF-1 cDNA clone prepared from mRNA of BALB/c 3T3 cells and the PLF-2 cDNA clone derived from mRNA of BALB/c placenta are presently unexplained. These differences may simply reflect DNA sequence differences between different BALB/c substrains. More interesting possibilities are the presence of multiple proliferin genes or mutational changes in 3T3 cells that have been functionally selected as the cell line emerged from primary embryonic fibroblasts.

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